(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification[7]: **A61K 45/00**, A61L 31/16, A61P 9/10, A61K 31/7105, 38/17, 39/395

(21) International Application Number: PCT/US02/34120

(22) International Filing Date: 23 October 2002 (23.10.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/335,637	24 October 2001 (24.10.2001)	US
60/341,359	17 December 2001 (17.12.2001)	US
10/128,166	23 April 2002 (23.04.2002)	US

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(81) **Designated States** *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZM, ZW.

(84) **Designated States** *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— with international search report
— before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) **Title:** TREATING VASCULAR DISEASE BY INHIBITING TOLL-LIKE RECEPTOR-4

(57) **Abstract:** Methods included herein describe the treatment of atherosclerosis and other vascular diseases such as thrombosis, restenosis after angioplasty and/or stenting, and vein-graft disease after bypass surgery, by inhibition of the expression or biologic activity of Toll-like receptor-4 (TLR-4). Also included is an intravascular device coated with a compound that inhibits TLR-4; thereby imparting an improved efficacy to the device. TLR-4 cell signal transduction is at least partially responsible for the manifestation, continuation, and/or worsening of atherosclerosis and other forms of vascular disease. The present invention provides several means with which to inhibit this signal transduction pathway.

WO 03/035110 A1

TREATING VASCULAR DISEASE BY INHIBITING TOLL-LIKE RECEPTOR-4

FIELD OF THE INVENTION

This invention relates to methods for inhibiting the biological activity of Toll-like

5   receptor-4 ("TLR-4"), and, in particular, to methods for treating vascular disease by inhibiting the

expression or signaling by TLR-4.

BACKGROUND OF THE INVENTION

Heart disease remains the leading cause of death worldwide, accounting for nearly 30% of

10   the annual total (i.e., approximately 15 million people). Heart and vascular disease debilitate

many more individuals every year. For many, atherosclerotic disease is a life-long process; it may

possess an initial stage in childhood, without clinical manifestation until middle age or later. Its

development has been repeatedly linked to unhealthy lifestyles (e.g., tobacco use, unbalanced diet,

and physical inactivity). Much progress has been made in the detection and treatment of various

15   forms of heart and vascular disease, but preventative measures and assorted treatment regimens

are usually incapable of halting or curing the underlying disease condition.

Experimental work over the past decade has linked inflammation of the blood vessel wall

to atherogenesis, restenosis, and plaque disruption. The precise triggers for inflammation are not

known, but it is believed that some triggers may include modified lipoproteins and various local

20   or distant infections. A potential role for infection in the development of atherosclerosis has been

considered; specific infectious agents, such as *Chlamydia pneumoniae* ("*C. pneumoniae*"), have

been suggested as playing a role in the progression and/or destabilization of atherosclerosis.

Recent studies suggest that chlamydia lipopolysaccharide ("cLPS") induces foam-cell

formation, whereas its heat-shock protein ("cHSP-60") induces oxidative modification of low-

25   density lipoproteins ("LDL"). M.V. Kalayoglu and G.I. Byrne, "*Chlamydia pneumoniae*

component that induces macrophage foam cell formation is chlamydial lipopolysaccharide,"
Infect. & Immunity 66:5067-5072 (1998); G.I. Byrne and M.V. Kalayoglu, "*Chlamydia*
pneumoniae and atherosclerosis: Links to the disease process," *Amer. Heart Journal* 138:S488-
S490 (1999). cHSP-60 has been implicated in the induction of deleterious immune responses in
human chlamydial infection and has been found to co-localize with infiltrating macrophages in
atheroma lesions. A.G. Kol *et al.*, "Chlamydial heat shock protein 60 localizes in human
atheroma and regulates macrophage tumor necrosis factor alpha and matrix metalloproteinase
expression," *Circulation* 98:300 (1998). Collectively, these data support a potential role for *C.*
pneumoniae in the development and progression of atherosclerosis and suggest that this organism
may indeed play an active role in atheroma development. However, available data underscore the
current lack of an understanding of the molecular mechanisms that link *C. pneumoniae* infections
to innate immunity and trigger the signals for enhanced inflammation and atherogenesis. Absent
such an understanding, it is quite difficult to develop a useful mechanism for treating vascular
disease based on these data.

Although precise triggers for inflammation in atherosclerosis are not fully understood,
hypercholesterolemia, modified lipoproteins, and infection with organisms such as *C. pneumoniae*
and others have been implicated. There is evidence that *C. pneumoniae* infection can accelerate
the progression and facilitate the induction of atherosclerosis in cholesterol-fed rabbits and
genetically modified atherosclerosis prone mice. Without a clear understanding of the mechanism
that controls this system, however, these data may not provide the basis for a treatment or cure for
atherosclerosis. J.B. Muhlestein *et al.*, "Infection with *Chlamydia pneumoniae* accelerates the
development of atherosclerosis and treatment with azithromycin prevents it in a rabbit model,"
Circulation 97:633-636 (1998); T.C. Moazed *et al.*, "Murine models of *Chlamydia pneumoniae*
infection and atherosclerosis," *J. Infect. Dis.* 175:883-890 (1997); T.C. Moazed *et al.*, "*Chlamydia*
pneumoniae infection accelerates the progression of atherosclerosis in Apolipoprotein E-deficient

mice," *J. Infect. Dis.* 180:238-241 (1999); L.A. Campbell and C.C. Kuo, "Mouse models of

Chlamydia pneumoniae infection and atherosclerosis," *Am. Heart J.* 138:S516-S518 (1999); K.

Laitinen *et al.*, "*Chlamydia pneumoniae* infection induces inflammatory changes in the aortas of

rabbits," *Infect & Immunity* 65:4832-4835 (1997).

5   The concept of *C. pneumoniae*-induced atherogenesis is strengthened by the finding that

antibiotic therapy against chlamydia prevents acceleration of atherosclerosis in the rabbit model.

Ingalls *et al.* have suggested lipopolysaccharide ("LPS"), and Kol *et al.* have implicated HSP-60

as the triggers for chlamydia-induced inflammatory responses. R.R. Ingalls *et al.*, "The

inflammatory cytokine response to *Chlamydia trachomatis* infection is endotoxin mediated,"

10   *Infect & Immun.* 63:3125-3130 (1995); A. Kol *et al.*, "Chlamydial and human heat shock protein

60s activate human vascular endothelium, smooth muscle cells and macrophages," *J Clin Invest*

103:571-577 (1999); A. Kol *et al.*, "Heat shock protein (HSP)60 activates the innate immune

response," *The J of Immunol.* 164:13-17 (2000). To date, however, the precise molecular

mechanisms by which infections such as *C. pneumoniae* contribute to the progression of

15   atherosclerosis and the links among lipids, microbial antigens, and innate immune and

inflammatory responses are not well understood.

One recent study, however, indicated that HSP-60 induces smooth muscle cell

proliferation *in vitro*; smooth muscle cell proliferation being directly related to atherogenesis.

Sasu et al., "Chlamydia pneumoniae and Chlamydial Heat Shock Protein 60 Stimulate

20   Proliferation of Vascular Smooth Muscle Cells via Toll-Like Receptor 4 and p44/p42 Mitogen-

Activated Protein Kinase Activation," *Circ. Res.* 89:244-250 (2001). The study showed that

smooth muscle cell proliferation was blocked or severely hampered by anti-TLR-4 antibodies.

This finding suggests that HSP-60 also causes smooth muscle cell proliferation via a TLR-4

pathway.

The introduction of surgical and percutaneous arterial revascularization to treat atherosclerosis has profoundly altered the clinical management of disease, but has also brought unanticipated problems and unanswered questions. Surgical, and especially percutaneous revascularization, may elicit an exaggerated healing response, which in many respects is similar to

5   the development of *de novo* atherosclerotic lesions. This "response to injury" is more proliferative in nature than *de novo* lesion formation, but may nevertheless lead to restenosis, or even late or abrupt vessel closure, and may ultimately result in a failed revascularization attempt. For this and additional reasons, long-term clinical studies have documented improved outcomes only in select patient subgroups; for those with stable angina pectoris, coronary intervention

10   remains merely palliative, and does not alter the progression or outcome of the underlying causative disease process.

With balloon coronary angioplasty, restenosis rates of 30%-40% or more have been documented, and certain lesion sites and patient subgroups have been found to be particularly susceptible to restenosis. Intensive research efforts into the cause of restenosis have yielded

15   considerable insight, but as yet no unequivocal treatment has been identified to eliminate the problem. Technical innovations in revascularization equipment and techniques have shown some success, but even this has been of limited efficacy. In particular, the development of the intracoronary stent markedly reduced the incidence of restenosis. With proper stent placement techniques, restenosis rates have been reduced to roughly 15%-30%, so intracoronary stent

20   placement has largely supplanted balloon angioplasty alone as the interventional coronary treatment of choice. Still, given the rapid proliferation and acceptance of intracoronary stenting, even a 15%-30% restenosis rate results in a very large number of patients in whom the revascularization attempt has been unsuccessful, and for whom other treatment strategies have not been sufficiently effective. Often, the same patient may need multiple separate interventions, and

25   ultimately these may not be successful.

Since the arterial response to injury is predominantly mitogenic and neoproliferative in nature, intracoronary irradiation (or intracoronary brachytherapy) has been developed and deployed to attempt to reduce further the number of patients who restenose following coronary intervention. Intracoronary brachytherapy has also met with limited success, however, and has

5   brought with it two new manifestations of the disease as a side effect: geometric miss and late in-stent thrombosis. It appears likely that these two effects will significantly limit the efficacy of intracoronary brachytherapy as a definitive treatment for restenosis. Thus, a need remains for an effective way to limit or eliminate restenosis following coronary stent placement. Alternatively, if intracoronary brachytherapy is to achieve unequivocal effectiveness in eliminating restenosis

10   following stent placement, a solution to late in-stent thrombosis and geometric miss must be found.

Conventional treatments for vascular disease have substantial drawbacks; many are only partially effective, and few provide a true cure for associated conditions. There remains a clear need in the art for a method of preventing, treating, and curing vascular disease, including

15   atherosclerosis. There remains a further need in the art for improvements to present stent technology, whereby one can minimize the chance of restenosis.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide methods for inhibiting the biological

20   activity of TLR-4, as, for example, by inhibiting its expression or signaling. It is a further object of the invention to provide methods of treating those diseases in which inhibiting TLR-4 would have a beneficial effect. Such diseases include, for example, vascular disease such as atherosclerosis and thrombosis, restenosis after angioplasty and/or stenting, and vein-graft disease after bypass surgery.

A first embodiment of the invention is directed to a method of inhibiting TLR-4 by administering to a mammal recombinant viral vectors (e.g., adenovirus, adeno-associated virus, retroviruses, lentiviruses, or other viral vectors) that deliver genes expressing antisense TLR-4 RNA; doing so inhibits the expression of TLR-4, thereby inhibiting its biological activity. An

5   optimal amount of viral particles and an effective and convenient route to administer it (e.g., by administering it intravenously or intramuscularly) can readily be determined by one of ordinary skill in the art of microbiology.

A second embodiment of the present invention is directed to a method of inhibiting TLR-4 signaling by inducing *in vivo* production of a high affinity soluble TLR-4 protein that competes

10   for non-bound HSP-60, LPS, and other ligands that are molecularly configured to operatively interact with a TLR-4 receptor. The TLR-4 protein most preferably lacks the TLR-4 signal transduction domain, or at least a sufficient amount of the TLR-4 signal transduction domain such that the TLR-4 protein is unable to participate in TLR-4 signal transduction. The method involves delivering viral vectors to produce an amount of soluble TLR-4 or its derivatives that is sufficient

15   to reduce the amount of HSP-60, LPS, or other ligands that are molecularly configured to operatively interact with a TLR-4 receptor, thereby inhibiting the TLR-4 signaling pathway.

A third embodiment of the present invention is directed to a method of inhibiting TLR-4 signaling with somatic-cell gene therapy. According to this method, one administers a ribozyme-viral (adeno, adeno-associated, lentiviral or other) vector against TLR-4 mRNA in a mammal.

20   The method utilizes a hammerhead ribozyme expression cassette in a viral backbone. Ribozymes have sequence-specific endoribonuclease activity, which makes them useful for sequence-specific cleavage of mRNAs and further inhibition of gene expression. Ribozyme therapy is widely regarded as a new and potential pharmaceutical class of reagent to treat a number of medical disorders. A desired quantity or the length of expression of the ribozyme-viral vector can be

25   readily determined without undue experimentation, as can the most effective and convenient route

of administering it. Ribozyme-viral vectors against TLR-4 mRNA permit one to uniquely assess
the contribution of TLR-4 mediated cell-signaling to vascular physiology, and to therapeutically
intervene in the pathology such signaling causes.

A fourth embodiment of the present invention provides a non-viral method to inhibit the
expression of TLR-4. This method involves antisense therapy using oligodeoxynucleotides
("ODN") that inhibit the expression of the TLR-4 gene product by specific base pairing of single
stranded regions of the TLR-4 mRNA. The method involves synthesis of ODN complimentary to
a sufficient portion of TLR-4 mRNA. The method further provides an effective amount of ODN
to inhibit the TLR-4 signaling pathways in a mammal.

A fifth embodiment of the present invention provides a method to inhibit the expression of
TLR-4 by RNA interference ("RNAi"). This method involves the use of double-stranded RNA
("dsRNA") that are sufficiently homologous to a portion of the TLR-4 gene product such that the
dsRNA degrades mRNA that would otherwise affect the production of TLR-4. A well-defined
21-base duplex RNA, referred to as small interfering RNA ("siRNA"), may operate in
conjunction with various cellular components to silence the TLR-4 gene product with sequence
homology.

A sixth embodiment of the present invention provides a method to inhibit the TLR-4 cell-
signaling pathway by peptide mimetics. This method involves the introduction of small peptides
(i.e., peptides of approximately 10-20 amino acids) that bind to TLR-4 ligands, thereby preventing
proper TLR-4 ligands from binding to TLR-4 or associated receptors (e.g., MD2). In this manner,
the TLR-4 cell-signaling pathway may be blocked from signal transduction, because the proper
TLR-4 ligands are unable to bind correctly to TLR-4 or its associated receptors.

A seventh embodiment of the present invention provides a method to inhibit the
expression of TLR-4 through the introduction an anti-TLR-4 antibody. Such an antibody may be
delivered to a mammal through any conventional mechanism in an amount effective to inhibit the

TLR-4 signaling pathways in a mammal; the mechanism of delivery and quantity of antibody

necessary for inhibiting TLR-4 expression both being readily ascertainable without undue

experimentation.

Other features and advantages of the invention will become apparent from the following

5   detailed description, taken in conjunction with the accompanying drawings, which illustrate, by

way of example, various features of embodiments of the invention.

BRIEF DESCRIPTION OF THE FIGURES

The file of this patent contains at least one drawing executed in color. Copies of this

10   patent with color drawing(s) will be provided by the Patent and Trademark Office upon request

and payment of the necessary fee.

Fig. 1 is executed in color. Fig. 1a is a histologic depiction of TLR-4 immunoreactivity

(brown) within the lipid core of an atherosclerotic plaque in the aortic sinus of an apolipoprotein

E-deficient mouse. Figs. 1b and 1c depict the histology of macrophage (brown) and smooth

15   muscle cell (red) immunoreactivity, respectively, in the serial section of the same aortic sinus.

Fig. 1d depicts Rabbit IgG staining for a negative control. Fig. 1e depicts a lack of TLR-4

immunoreactivity in the non-atherosclerotic aortic mouse sinus.

Fig. 2 is executed in color, and is a series of photomicrographs indicating TLR-4

expression in human atherosclerotic lipid-rich plaques, and a lack of such expression in fibrous

20   plaques. Fig. 2a depicts an atherosclerotic plaque stained brown with rabbit anti-human TLR-4

antiserum. Fig 2b depicts a negative control where the primary antibody was replaced by rabbit

IgG. Fig 2c depicts TLR-4 immunoreactivity (brown). Fig. 2d depicts a double immunostain of

TLR-4 (brown) and macrophages (red), demonstrating co-localization. Fig. 2e depicts

macrophage immunoreactivity (red), under a higher magnification. Fig. 2f depicts TLR-4

25   immunoreactivity (brown), under a higher magnification. Fig. 2g depicts macrophage (red) along

with TLR-4 (brown) immunoreactivity, under a higher magnification. Fig. 2h depicts a lack of

immunoreactivity of TLR-4 in a fibrous plaque. Fig. 2i depicts smooth muscle cell alpha actin

immunoreactivity (red) without TLR-4 immunoreactivity (brown) upon double-staining. Fig. 2j

depicts a lack of immunoreactivity of macrophages in a fibrous plaque. Fig. 2k depicts a negative

5   control using pre-absorption of the antiserum with the peptide. Fig. 2l depicts a normal mammary

artery with only minimal immunoreactivity of TLR-4 along the endothelial border.

Fig. 3 is not executed in color, and depicts the relative intensity of each band, at indicated

dosage levels, of TLR-4 expression when analyzed by reverse transcription polymerase chain

reaction ("RT-PCR"), relative to GAPDH expression in cultured human monocyte derived

10   macrophages that were stimulated with either native or oxidized LDL for five hours.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Methods of the present invention inhibit Toll-like receptor-4 ("TLR-4") activity and

expression by interfering with the production or biological activity of native TLR-4. One can use

15   these methods to treat any disease in which inhibiting TLR-4 activity has a beneficial effect on a

patient (e.g., ameliorating a disease, lessening the severity of its complications, preventing it from

manifesting, preventing it from recurring, merely preventing it from worsening, or a therapeutic

effort to affect any of the aforementioned, even if such therapeutic effort is ultimately

unsuccessful). Diseases are known in the art in which TLR-4 activity is known or suspected to

20   play a role in initiating, aggravating, or maintaining the pathological state that comprises the

disease. Atherosclerosis, restenosis, inflammation and other vascular diseases are examples.

Methods of the present invention may be used to treat any of these diseases.

In a preferred embodiment, methods of the present invention are used to inhibit

atherosclerosis, transplant atherosclerosis, vein-graft atherosclerosis, stent restenosis, and

25   angioplasty restenosis, and to thereby treat the cardiovascular diseases that atherosclerosis causes

(hereinafter "vascular diseases"). These methods may be used in any patient who could benefit from reducing atherosclerosis that is already present, from inhibiting atherosclerosis that has yet to form, or from both reducing existing atherosclerosis and inhibiting new atherosclerosis. Such patients include those suffering from, for example, angina pectoris and its subtypes (e.g., unstable

5 angina and variant angina); ischemias affecting organs such as the brain, heart, bone, and intestines, and conditions associated with the ischemias, such as stroke, transient ischemic attacks, heart attack, osteonecrosis, colitis, poor kidney function, and congestive heart failure; poor blood circulation to the extremities and the complications of poor blood circulation, such as slow wound healing, infections, and claudication; atherosclerosis itself, including restenosis following

10 angioplasty or stenting of atherosclerotic lesions; vein-graft atherosclerosis following bypass surgery; transplant atherosclerosis; and other diseases caused by or associated with atherosclerosis.

TLR-4 may be encoded by the RNA sequence set forth herein as SEQ ID NO. 6. Inhibition of this RNA, or those substantially similar to it, may correspondingly inhibit the

15 biological activity of TLR-4. Thus, various methods of the present invention are directed to inhibiting the expression of TLR-4 RNA.

The present invention contemplates a variety of TLR-4 inhibitors that are employed to inhibit the biological activity of TLR-4. These inhibitors may be administered to a mammal by any suitable means, such as those set forth in the various ensuing embodiments. Such inhibitors

20 may include any compound, pharmaceutical, or other composition that affects an inhibition of the biological activity of TLR-4. Such a composition may be administered to a mammal in an effective amount and by any suitable means, including, but not limited to, orally, topically, intraveneously, intramuscularly, via a surgical device, such as a catheter, or via an implantable mechanism, such as a stent.

A first aspect of the present invention includes somatic cell gene transfer utilizing viral vectors containing TLR-4 gene sequences that express antisense RNA. Appropriate viral vectors that can express antisense TLR-4 RNA include expression vectors based on recombinant adenoviruses, adeno-associated viruses, retroviruses or lentiviruses, though non-viral vectors may

5   be used, as well. An ideal vector for TLR-4 antisense gene transfer against atherosclerosis and angioplasty/stent-induced restenosis in mammals has the following attributes: (1) high efficacy of *in vivo* gene transfer; (2) recombinant gene expression in dividing as well as nondividing cells (the baseline mitotic rate in the coronary artery wall is <1% even in advanced lesions); (3) rapid and long-lived recombinant gene expression; (4) minimal vascular toxicity from inflammatory or

10   immune responses; (5) absence of baseline immunity to the vector in the majority of the population; and (6) lack of pathogenicity of viral vectors. This is not to say that a vector must have all of these attributes; indeed, many useful vectors will not.

In a preferred embodiment of the invention, one employs adenovirus serotype 5 ("Ad5")-based vectors (available from Quantum Biotechnology, Inc., Montreal, Quebec, Canada) to

15   deliver and express TLR-4 gene sequences expressing antisense RNA in cultured macrophages and vascular smooth muscle cells and in atherosclerosis-prone mice and swine. The recombinant Ad5 vectors have several advantages over other vectors such as liposomes and retroviruses. Unlike retroviral vectors, proliferation of the target cell is not required for infection by adenovirus vectors and thus, Ad5 vectors can infect cells *in vivo* in their quiescent state. Ad5 vectors are

20   capable of infecting a number of different tissues although the transduction efficiency can vary according to the cell type. However, Ad5 vectors as a means of *in vivo* gene delivery have several drawbacks: (1) gene expression from cells transduced with the Ad5 vector is often transient due to the elimination of the Ad5-transduced cells by the host immune system; (2) Ad5 vectors may generate some toxicity to human recipients as observed in human clinical trials in cystic fibrosis

25   patients; and (3) initial administration of Ad5 vectors produces blocking antibodies to the vectors,

thus repeated administrations of the adenoviral vector may not be effective. Even with these

limitations, methods of the present invention utilize rAd5-mediated transfer of the TLR-4

sequence expressing antisense RNA. Using RT-PCR, a portion of TLR-4 is isolated and cloned

upstream to the human cytomegalovirus ("CMV") major immediate early promoter-enhancer in a

5   direction to generate antisense TLR-4 RNA. The use of recombinant Ad5 vectors provides proof

of the principle that adenovirus-mediated gene therapy might be particularly well suited as an

adjunct to coronary angioplasty, since even temporary inhibition of smooth muscle cell

proliferation might suffice to limit the formation of restenotic lesions.

A second aspect of the present invention provides a gene therapeutic method to produce

10   high levels of soluble forms of membrane bound TLR-4 that compete for non-bound HSP-60,

LPS, and other ligands that are molecularly configured to operatively interact with a TLR-4

receptor, but lack at least a substantial portion of the TLR-4 signal transduction domain.

As with other types of disease, therapeutic strategies to treat atherosclerotic disease entail

treatment for an extended period of time ranging from months to years. Prolonged and efficient

15   transgene transcription from heterologous promoters is a major consideration for gene therapies.

The inclusion of a CMV promoter to drive expression of soluble TLR-4 in the present invention

has been popularly used to express a variety of genes. It is, however, often subject to epigenetic

silencing as are most promoters and transgenes. In an attempt to circumvent this problem, a

variety of promoter expression strategies can be used to optimize the *in vivo* production of the

20   soluble TLR-4 in the present invention.

Efficient gene expression in viral vectors depends on a variety of factors. These include

promoter strength, message stability and translational efficiency. Each of these factors must be

explored independently to achieve optimal expression of a soluble TLR-4 gene. Applications of

other promoter/enhancer variants to increase and optimize the expression of soluble TLR-4 *in*

25   *vitro* as well as *in vivo* are included within the scope of this invention. These include promoters

or enhancers stronger than CMV that exhibit inducibilty such as tetracycline inducible promoters. Promoters/enhancers with tissue-specific functions that target, for example, vascular endothelial or smooth muscle tissue, and that produce sufficient amounts of soluble TLR-4 or its derivatives for a time and under condition sufficient to reduce the amount of TLR-4 ligand and thereby

5   inhibit the TLR-4 function may also be included. Levels and persistence of soluble TLR-4 expression can be compared with those obtained from the CMV promoter.

A third aspect of the present invention contemplates a somatic cell gene therapeutic method by administering a ribozyme-viral (adeno, adeno-associated or lentiviral) or non-viral vector against TLR-4 mRNA in a mammal, and in particular in humans for treating the conditions

10   referred to above. The method involves development of a hammerhead ribozyme expression cassette that targets a sequence of TLR-4 mRNA. Ribozymes are sequence-specific endoribonucleases that catalytically cleave specific RNA sequences, resulting in irreversible inactivation of the target mRNA, thereby inhibiting the gene expression. T. Cech, "Biological catalysis by RNA," *Ann Rev Biochem.* 55:599-629 (1986); J.J. Rossi, "Therapeutic ribozymes:

15   principles and applications," *Bio Drugs* 9:1-10 (1998). Ribozymes offer advantages over antisense ODN. For instance, rybozymes possess higher catalytic activity than ODN; a comparatively smaller quantity of rybozyme-containing active is thus required for inhibition of gene expression. Ribozymes can be delivered exogenously or can be expressed endogenously with the use of appropriate promoters in a viral vector. Methods of the present invention utilize a

20   hammerhead ribozyme directed to human TLR-4 mRNA. Desired quantity or the length of expression of the ribozyme-viral or non-viral vector can readily be determined by routine experimentation, as can the most effective and/or convenient route of administration.

In a fourth aspect of the present invention, there is provided a non-viral method to inhibit the expression of TLR-4. This method involves synthesis of pentadecamer ("15-mer") ODN

25   corresponding to the sense and antisense sequence of human TLR-4 mRNA. Pentadecamer ODN

are known to bind strongly to single-stranded regions of target mRNA. D. Jaskuski *et al.*, "Inhibition of cellular proliferation by antisense oligonucleotide to PCNA cyclin," *Science* 240:1544-1548 (1988). Such strong binding may correspondingly result in strong inhibition of the translation of mRNA.

5   In a preferred method of the present invention, ODN are synthesized on a nucleic acid synthesizer, such as the EXPIDITE Nucleic Acid Synthesizer (available from Applied Biosystems, Inc., Rockville, MD) and purified using standard protocols.

In a fifth aspect of the present invention, there is provided a method to inhibit the expression of TLR-4 by RNAi. This new approach to silencing a gene product by degrading a

10   corresponding RNA sequence is reportedly more effective than alternative gene silencing methodologies, including antisense and ribozyme-based strategies. The method involves the use of dsRNA that are sufficiently homologous to a portion of the TLR-4 gene product such that the dsRNA degrades mRNA that would otherwise affect the production of TLR-4. siRNA, a well-defined 21-base duplex RNA (obtained from Dharmacon Research, Inc., Boulder, CO), may

15   operate in conjunction with various cellular components to silence the TLR-4 gene product with sequence homology. RNAi is described in Hammond *et al.*, "Post-Transcriptional Gene-Silencing by Double-Stranded RNA," *Nature* 110-119 (2001); Sharp, P.A., "RNA interference - 2001," *Genes Dev.* 15:485-490 (2001); and Elbashir, *et al.*, "RNA interference is mediated by 21- and 22-nucleotide RNAs," *Genes Dev.* 15:188-200, each of which is incorporated by reference

20   herein in its entirety.

Efficient gene silencing may be achieved by employing siRNA duplexes which include sense and antisense strands each including approximately 21 nucleotides, and further paired such that they possess about a 19-nucleotide duplex region and about a 2-nucleotide overhang at each 3' terminus. Elbashir *et al.*, "Duplexes of 21-nucleotide RNAs mediate RNA interference in

25   cultured mammalian cells," *Nature* 411:494-498 (2001). It will be appreciated by one of skill in

the art of RNAi that alternately sized sense or antisense strands and/or variations on the size of the duplex and the overhang region that comprise them may be suitable for use with the methods of the present invention, and are contemplated as being within the scope thereof. Such appropriate alternate sizes may be readily ascertained without undue experimentation by one possessing such

5   skill.

Furthermore, the inclusion of symmetric 3'-terminus overhangs may aid in the formation of specific endonuclease complexes ("siRNPs") with roughly equivalent ratios of sense and antisense target RNA cleaving siRNPs. It is believed that the antisense siRNA strand is responsible for target RNA recognition, while the 3'-overhang in the sense strand is not involved

10   in this function. Therefore, in a preferred embodiment, the UU or dTdT 3'-overhang of an antisense sequence is complementary to target mRNA, however the symmetrical UU or dTdT 3'-overhang of the sense siRNA oligo need not correspond to the mRNA. Deoxythymidines may be included in either or both 3'-overhangs; this may increase nuclease resistance. However, siRNA duplexes that include either UU or dTdT overhangs may be equally resistant to nuclease.

15   The siRNA duplexes used in accordance with the present invention may be introduced to a cell via an appropriate viral or non-viral vector. Such vectors include those described above with regard to the somatic gene cell transfer embodiment of the present invention.

In a sixth aspect of the present invention, a method of inhibiting the TLR-4 cell-signaling pathway by peptide mimetics is provided. This method involves the introduction of small

20   peptides (i.e., peptides of approximately 10-20 amino acids) that bind to TLR-4 ligands, thereby preventing these ligands from binding to a TLR-4 or associated receptor (e.g., MD2). TLR-4 is generally found on a cell surface substantially adjacent to an MD2 receptor. It is believed that, in order to initiate TLR-4 cell signal transduction, a TLR-4 ligand must bind simultaneously to the TLR-4 receptor as well as the adjacent MD2 receptor. Binding to only one of these receptors is

25   insufficient to propagate TLR-4 cell signal transduction. Bulut *et al.*, "Chlamydial Heat Shock

Protein 60 Activates Macrophages and Endothelial Cells Through Toll-like Receptor 4 and MD2 in a MyD88-Dependent Pathway," *J. Immunol.*, 168:1435-1440.

The MD2 receptor is a protein constructed of approximately 133 individual amino acids. Short, overlapping segments (e.g., approximately 10-20 amino acids in length) of the MD2

5   receptor molecule may be separated to test which individual segments effect TLR-4 cell signal transduction by binding to a TLR-4 ligand. Segments are overlapping insofar as a portion of one end of one segment separated for testing corresponds to a portion of one end of a second segment separated for testing. Following separation, the segments are duplicated and tested to determine which comprise at least a portion of the MD2 receptor that binds to a TLR-4 ligand, such as

10   cHSP60, LPS, or other ligands that are molecularly configured to operatively interact with a TLR-4 receptor. A segment suitable for use in accordance with the method of the present invention comprises at least a portion of the MD2 receptor that binds to a TLR-4 ligand, such that the administration of a sufficient amount of individual copies of this segment will hinder TLR-4 signal transduction. Once administered, segments preferably bind to the MD2 binding sites of the

15   TLR-4 ligands, thereby preventing the ligands from binding to the corresponding sites on the MD2 receptor. This may significantly hinder TLR-4 cell signal transduction.

The same process may be implemented to identify a segment of the TLR-4 receptor that may similarly hinder TLR-4 signal transduction. Short, overlapping segments (e.g., approximately 10-20 amino acids in length) of the TLR-4 receptor molecule may be separated to

20   test which individual segments effect TLR-4 cell signal transduction by binding to a TLR-4 ligand. Following separation, the segments are duplicated and tested to determine whether the segment comprises at least a portion of the TLR-4 receptor that binds to a TLR-4 ligand, such as cHSP60, LPS, or other ligands that are molecularly configured to operatively interact with a TLR-4 receptor. A segment suitable for use in accordance with the method of the present invention

25   comprises at least a portion of the TLR-4 receptor that binds to a TLR-4 ligand, such that the

administration of a sufficient amount of individual copies of this segment will hinder TLR-4

signal transduction. Once administered, segments preferably bind to the binding sites of the TLR-

4 ligands, thereby preventing the ligands from binding to the corresponding sites on the TLR-4

receptor. This may significantly hinder TLR-4 cell signal transduction.

5   In accordance with the method of the present invention, a segment that does, in fact,

include at least a portion of the MD2 or TLR-4 receptor that binds to a TLR-4 ligand may be

administered to a patient. Segments that include a portion of the MD2 receptor, segments that

include a portion of the TLR-4 receptor, or combinations thereof may be administered. Moreover,

administration may be performed by any suitable means, including via an oral form, such as a

10   capsule, tablet, solution, or suspension; an intravenous form; an injectable form; an implantable

form, such as a stent coating, a sustained release mechanism, or a biodegradable polymer unit; or

any other suitable mechanism by which an active or therapeutic agent may be delivered to a

patient. The dosage may similarly be determined in accordance with the selected form of

administration, the level of which may be readily ascertained without undue experimentation, as

15   can the most suitable means of administration.

In a seventh aspect of the present invention, a method of inhibiting TLR-4 expression

through the introduction an anti-TLR-4 antibody is provided. Any suitable anti-TLR-4 antibody

may be used in conjunction with this aspect of the present invention, including, but in no way

limited to, anti-TLR-4 antibodies, and any suitable derivatives thereof, equivalents thereof, or

20   compounds with active sites that functions in a manner similar to anti-TLR-4 antibodies, whether

those compounds are naturally occurring or synthetic (all hereinafter included within the term

"anti-TLR-4 antibody").

An appropriate quantity of an anti-TLR-4 antibody necessary to affect the method of the

present invention, and the most convenient route of delivering the same to a mammal may be

25   determined by one of ordinary skill in the art, without undue experimentation. Furthermore, it

will be readily appreciated by one of such skill that an anti-TLR-4 antibody may be formulated in a variety of pharmaceutical compositions, any one of which may be suitable for use in accordance with the method of the present invention.

Such an antibody may be delivered to a mammal through any conventional mechanism in an amount effective to inhibit the TLR-4 signaling pathways in a mammal; the mechanism of delivery and quantity of antibody necessary for inhibiting TLR-4 expression both being readily ascertainable without undue experimentation.

The vascular delivery of TLR-4 inhibiting compositions composed in accordance with any of the various embodiments of the present invention can be accomplished by any of a wide range of local delivery devices and methods. K. L. March, "Methods of local gene delivery to vascular tissues," *Semin Intervent Cardiol*, 1:215-223 (1996). Local delivery is preferred because, for those compositions that include a viral or non-viral vector, site-specific delivery may result in maximal therapeutic efficacy with minimal systemic side effects. These local delivery devices typically entail an endovascular or "inside-out" approach, whereby therapeutic agents are delivered to the target site via intravascular catheters or devices. Although gene transfer is demonstrated for each device, most studies of catheter-based gene transfer reveal low efficiency, rapid redistribution of the infused material, and escape of the infusate into the systemic circulation.

Recently, several devices with modified needles capable of direct injection into interstitial tissue of either myocardium or vasculature have been described. One such approach to local drug delivery is via the nipple balloon catheter, such as the INFILTRATOR® (available from InterVentional Technologies, Inc., San Diego, CA), although any appropriate catheter may be used. Methods of the present invention utilize the INFILTRATOR® for intramural delivery of small volumes of high-titer rAd5, where such a viral vector is appropriate. The INFILTRATOR® catheter offers improved local gene delivery by placing vector particles directly and deeply within

the vascular wall. The INFILTRATOR® catheter is designed to provide direct intramural delivery of agents by mechanical access into the media and inner adventitia, which is achieved using sharp-edged injection orifices mounted on the balloon surface. P. Barath *et al.*, "Nipple balloon catheter," *Semin Intervent Cardiol*, 1:43 (1996). This catheter has been used clinically.

5   G. S. Pavlides *et al.*, "Intramural drug delivery by direct injection within the arterial wall: first clinical experience with a novel intracoronary delivery-infiltrator system," *Cathet Cardiovasc Diagn*, 41:287-292 (1997). Further, the INFILTRATOR® has been demonstrated to yield enhanced local transduction efficiency by adenoviral vectors compared with that which may be achieved by endoluminal delivery. T. Asahara *et al.*, "Local delivery of vascular endothelial

10   growth factor accelerates reendothelialization and attenuates intimal hyperplasia in balloon-injured rat carotid artery," *Circulation*, 91:2793-2801 (1995).

Methods of the present invention also utilize a perivascular or "outside-in" approach of drug delivery in the vessel wall by modifying the procedure applied in periadventitial carotid injury in a mouse, as described in Example 10 below. Oguchi S, *et al.* "Increased intimal

15   thickening after arterial injury in hypercholesterolemic apolipoprotein E-deficient mice: finding a novel method," *Circulation (supp.)* I-548:3066 (1997); P. Dimayuga *et al.*, "Reconstituted HDL containing human apolipoprotein A-1 reduces VCAM-1 expression and neointima formation following periadventitial cuff-induced carotid injury in apo E null mice" *Biochem Biophys Res Commun.* 264:465-468, (1999).

20   By directly targeting the genes involved via gene therapeutic approaches, methods of the present invention may be used in stent coatings that eliminate or substantially reduce restenosis following stent placement, as well as geometric miss and late in-stent thrombosis following intracoronary brachytherapy. Methods of the present invention contemplate stents coated with TLR-4 inhibiting compositions. As these gene therapeutic agents may be used as coatings on

25   already existing stents, they may be deployed without increasing procedure time, and will not

require significant additional equipment, expertise, hospitalization or expense. This strategy should prove cost-effective in the long run since, if successful, it will diminish the need for repeat hospitalizations and additional intervention procedures. Outcomes should also be favorable, to the extent that the strategy is effective in minimizing clinical events associated with restenosis

5   following stent placement, and geometric miss and late in-stent thrombosis following intracoronary brachytherapy. Coated stents may eventually be implanted in all patients who are candidates for stents, since it is presently not possible to determine prior to the procedure which patients will suffer from restenosis or other complications associated with arterial injury following coronary intervention.

10   Since TLR-2 and TLR-4 play an important role in the innate immune and inflammatory response, we investigated the expression of these receptors, and found that TLR-4 exhibits preferential expression in lipid-rich and macrophage-infiltrated murine aortic and human coronary atherosclerotic plaques. The inventor's *in vitro* studies, described below, demonstrated basal expression of TLR-4 by macrophages, which was up-regulated by oxidized LDL ("ox-LDL").

15   While not wishing to be bound by any theory, these findings suggest a potential role for TLR-4 in lipid-mediated pro-inflammatory signaling in atherosclerosis. Moreover, as TLR-4 is a receptor that recognizes chlamydial antigens such as cLPS and cHSP-60, endotoxin, and other ligands that are molecularly configured to operatively interact with a TLR-4 receptor it may provide a molecular link between chronic infection, inflammation, and atherosclerosis.

20   The pro-inflammatory signaling receptor TLR-4 is expressed in lipid-rich, macrophage-infiltrated atherosclerotic lesions of mice and humans. Further, TLR-4 mRNA in cultured macrophages is up-regulated by ox-LDL but not native LDL ("N-LDL"). Together, these findings suggest that enhanced TLR-4 expression may play a role in inflammation in atherosclerosis.

Cells of the innate immune system, such as macrophages, have the ability to recognize common and conserved structural components of microbial origin by pattern recognition receptors. The human homologue of *Drosophila* Toll, TLR-4, is a pattern recognition receptor, which activates NF-κB, and up-regulates a variety of inflammatory genes in response to microbial

5   pathogens. Toll-like receptors play a fundamental role in the activation of innate immune responses and pathogen recognition. Further, activation of NF-κB is essential for the regulation of a variety of genes involved in the inflammatory and proliferative responses of cells critical to atherogenesis. Both NF-κB and genes regulated by NF-κB are expressed in atherosclerotic lesions. Since NF-κB activation leads to transcription of a number of pro-inflammatory genes

10   involved in athero-thrombosis, it may be that infectious agents and clamydial antigens such as LPS and/or HSP-60 contribute to enhanced and chronic inflammation by signaling through the TLR-4 receptor, which is up-regulated by ox-LDL.

The inventor's findings of increased expression of TLR-4 induced by ox-LDL suggests a potential mechanism for the synergistic effects of hypercholesterolemia and infection in

15   acceleration of atherosclerosis observed in experimental models and human epidemiologic observations. This provides new insight into the link among lipids, infection/inflammation and atherosclerosis.

EXAMPLE 1

20   Preparation of Mouse Tissue

Five apolipoprotein E-deficient ("apoE -/-") mice (C57BL/6J strain, aged 5 weeks, 18 to 20 grams; obtained from Jackson Laboratory, Bar Harbor, ME) were fed a high fat, high cholesterol (i.e., atherogenic) diet containing 42% (wt/wt) fat and 0.15% cholesterol from 6 weeks of age through the duration of the experiment. After anesthesia with ETHRANE (available from

25   Abbot Laboratories, Abbott Park, IL), the mice were sacrificed at 26 weeks of age, and their

hearts and proximal aortas (including ascending aorta, aortic arch and a portion of descending

aorta) were excised and washed in phosphate-buffered saline ("PBS") to remove blood. The basal

portion of the heart and proximal aorta were embedded in OCT compound using TISSUE-TEK

VIP (available from Sakura Finetek USA, Inc., Torrance, CA), frozen on dry ice and then stored

5   at -70°C until sectioning. Serial 10μm-thick cryosections (every fifth section from the lower

portion of the ventricles to the appearance of aortic valves, every other section in the region of the

aortic sinus, and every fifth section from the disappearance of the aortic valves to the aortic arch)

were collected on poly-D-lysine-coated slides (available from Becton Dickinson & Co., Franklin

Lakes, NJ). Sections were stained with Oil Red O and hematoxylin, and counterstained with Fast

10   Green (all available from Sigma Chemical Co., St. Louis, MO, "Sigma") for the identification of

atheromatous lesions, arterial wall calcification, and cartilaginous metaplasia. The presence of

calcium deposits was confirmed by the alizarin red S (available from Sigma) and von Kossa

techniques using representative sections.

15   EXAMPLE 2

Preparation of Human Tissue and Human Monocyte-Derived Macrophages

Human coronary artery specimens from nine autopsy cases were collected within 24 hours

of death, fixed with 10% formalin (available from Sigma) overnight and embedded in paraffin.

Five of the nine coronary artery specimens included lipid-rich plaques containing a well-defined

20   lipid-core covered by a fibrous cap, and the other four of the nine specimens included fibrous

plaques, which contained mostly extracellular matrix without a lipid-core. Normal mammary

artery specimens were also obtained from four additional autopsy cases. Five μm-thick sections

were cut and applied to slides for both hematoxyline-eosin and immunohistochemical staining.

Peripheral blood monocytes were isolated from whole blood of normal human subject by

25   FICOLL-PAQUE density gradient centrifugation (available from Pharmacia LKB Biotechnology,

Inc., Piscataway, NJ). Monocyte-derived macrophages were cultured in RPMI 1640 (available

from Sigma) containing 10% fetal calf serum ("FCS"), 100 U/ml penicillin, 100 μg/ml

streptomycin and 0.25 μg/ml amphotericin B for 5 days and then starved in the culture medium

without FCS but with 0.1% low endotoxin bovine serum albumin ("BSA") (obtained from

5   Sigma).

EXAMPLE 3

Immunohistochemistry

Frozen sections of the apoE -/- mouse aortic root were fixed with acetone for 5 minutes at

10   room temperature and then immunostained with rabbit anti-hTLR-4 immune serum (1:100;

obtained from Ruslan Medzhitov, Asst. Prof. of Immunobiology, Yale University, New Haven,

CT) following the instructions on the immunostaining kit available from DAKO (Carpinteria, CA,

"DAKO"). Rat anti-mouse macros Ab (1:500; available from Serotec, U.K.) were used as

macrophage marker. Colors were developed using the DAKO AES substrate system. Smooth

15   muscle cells were stained by a mouse anti-actin Ab conjugated with alkaline phosphatase (1:50,

available from Sigma). Colors were developed using VECTOR Red Alkaline Phosphatase

Substrate Kit I (obtained from Vector Laboratories, Inc., Burlingame, CA). Rabbit IgG or rabbit

serum was used as a negative control.

For human atherosclerotic plaques, following deparaffinization in graded alcohol, sections

20   were immunostained using rabbit anti-human TLR-4 and TLR-2 antiserum (1:100) raised against

extracellular peptide domains of TLR-4 and TLR-2 (available from Berkeley Antibody Company,

Richmond, CA). Following immunoperoxidase staining, the representative fields were

photographed. Cells were lysed in Laemmli buffer and separated with a 10% sodium dodecyl

sulphate polyacrylamide gel electrophoresis ("SDS-PAGE"). The protein was then transferred

25   onto a polyvinylidene difluoride membrane, and the membrane was probed with anti-TLR-2, anti-

TLR-4 antibodies, and prebleeds corresponding to each antibody (1:2,000). After incubation with

horseradish peroxidase-conjugated goat anti-rabbit antibody (available from Rockland

Immunochemicals for Research, Gilbertsville, PA), the membrane was developed with an

enhanced chemiluminescence ECL Western Blotting Detection Kit (available from Amersham

Pharmacia Biotech UK Ltd., Buckinghamshire, England). Pre-incubating the anti-TLR-4 serum

with TLR-4 peptide (SEQ ID NO. 5) was used to demonstrate specificity of the strain and rabbit

IgG or rabbit serum instead of primary antibody was used as a negative control.

EXAMPLE 4

Double Immunohistochemistry

Double immunostaining of human atherosclerotic plaques was performed using an

EnVision Doublestain System (available from DAKO). Following TLR-4 immunostaining, 3,3'-

diaminobenzadine (obtained from Sigma) was used as the peroxidase chromogenic substrate.

Mouse monoclonal anti-human CD68 antibody (360 µg/ml, 1:20 dilution; available from DAKO)

for macrophages and mouse monoclonal anti-human α-actin antibody (100 µg/ml, 1:100 dilution;

available from DAKO) for smooth muscle cells were used with Fast Red (available from Sigma)

as the alkaline phosphatase chromogenic substrate.

EXAMPLE 5

Preparation and Modification of Lipoproteins

Human N-LDL (obtained from Sigma) was dialyzed against isotonic phosphate saline

buffer (pH 7.4) to remove ethylenediamine tetraacetic acid ("EDTA") by using a 10,000

molecular weight cut-off SLIDE-A-LYZER dialysis cassette (obtained from Pierce Chemical Co.,

Rockford, IL). Ox-LDL was prepared by incubating 0.1 mg of LDL protein/ml with 5 µM of

copper sulfate ($CuSO_4$) for 24 hours at 37°C, and stopped by adding butylated hydroxytoluene

(2,6-di-*t*-butyl-*p*-cresol) (available from Sigma) to a final concentration of 0.1mM. Ox-LDL was

separated from $CuSO_4$ and equilibrated into the cell culture medium over a PD-10 column

(available from Pharmacia Fine Chemicals, Uppsala, Sweden). All reagents were endotoxin-free.

LPS levels of LDL preparations were confirmed with a chromogenic *Limulus* assay and contained

5   less than 0.3 pg of LPS/μg of LDL protein.

The extent of oxidation of the lipoprotein preparations was determined by a thiobarbituric

acid reactive substance ("TBARS") assay. Concentrated trichloroacetic acid was added to

aliquots of lipoprotein samples containing 1.5 mg of protein to give a final concentration of 5%.

An equal volume of 1% thiobarbituric acid was then added and the mixture was heated in a water

10   bath at 100°C for 20 min. After centrifugation to clarify the solution, the peak absorbance at 582

nm was read on a Beckman DB Spectrophotometer (available from Beckman Coulter, Inc.,

Fullerton, CA) against a buffer blank. The amount of thiobarbituric-reactive substance was

calculated from a standard curve, with malonaldehyde bis(dimethylacetal) (available from Sigma)

as the standard. The ox-LDL had 20-25 nM TBARS/mg of cholesterol.

15

EXAMPLE 6

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from resting N-LDL, ox-LDL stimulated human monocyte-

derived macrophage cells using an RNA Stat60 isolation reagent (obtained from Tel-test 'B', Inc.,

20   Friendswood, TX) following manufacturer's instruction and treated with RNase-free DNase I.

For RT reaction, the SUPERSCRIPT MMLV preamplification system (obtained from Life

Technologies, Inc., Gaithersburg, MD) was applied. PCR amplification was performed with TAQ

GOLD polymerase (obtained from Perkin Elmer, Foster City, CA) for 32 cycles at 95°C for 45s,

54°C for 45s, and 72°C for 60s (for TLR-2 and TLR-4). The oligonucleotide primers used for RT-

25   PCR for TLR-2 were SEQ ID NO. 1 and SEQ ID NO. 2, and for TLR-4 were SEQ ID NO. 3 and

SEQ ID NO. 4. Glyceraldehyde-3-phosphate dehydrogenase ("GAPDH") primers were obtained

from Clontech Laboratories, Inc. (Palo Alto, CA).

The TLR-2 and TLR-4 RT-PCR fragments were purified and sequenced to confirm the

identity of the fragments. Real-time quantitative PCR was performed on an iCycler Thermal

5   Cycler (obtained from Bio-Rad Laboratories, Inc., Hercules, CA) using an SYBR Green RT-PCR

Reagents kit (obtained from Applied Biosystems, Foster City, CA) and the TLR primers described

above. The semi-quantitative RT-PCR experiments were repeated with cells pretreated for 1 hour

with 15d-PGJ$_2$ (20 μM), proteasome inhibitor I (100 μM) (available from Affinity Bioreagents,

Inc., Golden, CO), or cycloheximide (10 μm/ml). Endothelial cells were pretreated with NF-κB

10   p65 antisense and sense oligonucleotides (30 μM) for 24-48 hours, three times before LPS

stimulation (50 ng/ml). For densitometry analysis, the intensity of the bands were measured by

Digital Science 1D Image Analysis Software (obtained from Eastman Kodak Co., Rochester, NY)

and normalized with GAPDH intensity.

15   EXAMPLE 7

                 TLR-4 is Expressed in Atherosclerotic Lesions of the ApoE -/- Mice

As depicted in Fig. 1, all five apoE -/- mice exhibited TLR-4 immunoreactivity in the

atherosclerotic lesions of the aortic root, which co-localized with macrophage immunoreactivity.

TLR-4 staining was absent in the normal vessels obtained from control C56BL/6J mice (Fig. 1e).

20   Mouse IgG staining was negative and pre-incubation of the tissue sections with the specific

peptide against which the anti-TLR-4 antiserum was generated completely blocked the TLR-4

staining in the apoE -/- vessels, indicating the specific nature of the TLR-4 immunostaining. No

TLR-2 immunoreactivity was observed in normal or atherosclerotic lesions (not shown).

EXAMPLE 8

TLR-4 is Expressed in Human Coronary Plaques

The human coronary atherosclerotic plaques were classified into lipid-rich plaques

containing a well-defined lipid-core covered by a fibrous cap (n=5), and fibrous plaques which

5   contained mostly extracellular matrix without a lipid-core (n=4). As depicted in Fig. 2, strong

TLR-4 expression (brown staining) was observed around the lipid core at the shoulder of lipid-

rich plaques where it co-localized with macrophage immunoreactivity. Incubation of the

antiserum with the peptide used to generate the primary antibody blocked TLR-4

immunoreactivity, confirming the specificity of the anti-TLR-4 antiserum. Double staining

10   showed close spatial co-localization of TLR-4 expression with macrophage immunoreactivity.

No TLR-4 immunoreactivity or macrophage immunoreactivity was found in fibrous plaques,

which demonstrated strong smooth muscle α-actin immunoreactivity. Normal mammary arteries

showed only minimal or no TLR-4 expression. TLR-2 immunoreactivity was absent in all

plaques while control staining was positive in THP-1 cells (not shown).

15

EXAMPLE 9

TLR-4 mRNA Regulation by Ox-LDL

Cultured human monocyte derived macrophages were stimulated with N-LDL or ox-LDL

for 5 hours. RT-PCR was performed for TLR-2 and TLR-4, and relative intensity was calculated

20   by densitometry as described in Faure *et al.*, at 2018-2024. As depicted in Fig. 3, RT-PCR

showed basal TLR-2 and TLR-4 mRNA expression by macrophages. The TLR-4 mRNA was

upregulated by ox-LDL in a dose-dependent manner and up to threefold, whereas N-LDL had no

effect. TLR-2 mRNA was not upregulated by ox-LDL.

EXAMPLE 10

Perivascular or "Outside In" Approach to Drug Delivery

ApoE -/- mice (20 weeks of age, 6 per group) were anesthetized, and the carotid artery

was exposed by making a small incision in the side of the neck. A section of artery was loosely

5   sheathed with a cuff made of a TYGON tube (3.0 mm long, 0.5 mm inner diameter; obtained

from Saint-Gobain Performance Plastics, Wayne, NJ). A biodegradable biocompatible polymeric

material, ATRIGEL (obtained from Atrix Laboratories, Ft. Collins, CO), a copolymer of

polylactic and polyglycolic acid, was used for the local delivery of viral particles. An 18% (w/w)

polymeric gel in PBS with 1×10^8 pfu of rAd5 (right carotid) or without rAd5 (left carotid) was

10   applied between the cuff and the vessel using a syringe and blunt cannula. The gel compound

used in the study was a free-flowing liquid below body temperature. When placed in an aqueous

environment at or above body temperature, the viscosity increases and the gel solidifies into a

viscous mass. Once applied to the artery *in vivo*, the polymer turns into a gel immediately on

contact and the gel is gradually resorbed in about 14 to 21 days, thereby providing potential use as

15   a drug depot.

WHAT IS CLAIMED IS:

1.   A system for inhibiting the biological activity of Toll-like receptor-4 (TLR-4)

comprising:

        an intravascular device; and

5   a therapeutic composition coated upon the intravascular device, the

therapeutic composition comprising a TLR-4 inhibitor.

2.   The system of claim 1, wherein the intravascular device is selected from the group

consisting of a catheter and a stent.

3.   The system of claim 1, wherein the TLR-4 inhibitor is selected from the group

10   consisting of a nucleic acid expressing antisense TLR-4 RNA, a nucleic acid encoding a

soluble TLR-4 protein, a nucleic acid encoding a hammerhead ribozyme that cleaves TLR-

4 mRNA, an antisense TLR-4 oligodeoxinucleotide (ODN), a nucleic acid expressing a

double stranded RNA (dsRNA) that is sufficiently homologous to a portion of a TLR-4

gene product such that the dsRNA is capable of inhibiting the encoding function of mRNA

15   that would otherwise cause the production of TLR-4, a protein sequence that corresponds

to at least a portion of a receptor that binds to a TLR-4 ligand during a TLR-4 signal

transduction event, and an anti-TLR-4 antibody.

4.   The system of claim 3, wherein the TLR-4 inhibitor is the nucleic acid expressing

antisense TLR-4 RNA.

20   5.   The system of claim 3, wherein the TLR-4 inhibitor is the nucleic acid encoding

the hammerhead ribozyme that cleaves TLR-4 mRNA.

6.   The system of claim 3, wherein the TLR-4 inhibitor is the antisense TLR-4 oligodeoxinucleotide (ODN).

7.   The system of claim 3, wherein the TLR-4 inhibitor is the anti-TLR-4 antibody.

8.   The system of claim 1, wherein the TLR-4 inhibitor is included within a vector.

9.   The system of claim 8, wherein the vector is selected from the group consisting of adenoviruses, adeno-associated viruses, retroviruses, lentiviruses, viral vectors, and non-viral vectors.

10.   The system of claim 8, wherein the vector is an adenovirus serotype 5-based vector.

11.   The system of claim 8, wherein the TLR-4 inhibitor is selected from the group consisting of a nucleic acid expressing antisense TLR-4 RNA, a nucleic acid encoding soluble TLR-4 protein, a nucleic acid encoding a hammerhead ribozyme that cleaves TLR-4 mRNA, and a nucleic acid expressing a double stranded RNA (dsRNA) that is sufficiently homologous to a portion of a TLR-4 gene product such that the dsRNA is capable of inhibiting the encoding function of mRNA that would otherwise cause the production of TLR-4.

12.   The system of claim 1, further comprising an amount of the therapeutic composition sufficient to inhibit a vascular disease.

13.   The system of claim 12, wherein the vascular disease is selected from the group consisting of atherosclerosis, transplant atherosclerosis, vein-graft atherosclerosis, thrombosis, restenosis, stent restenosis, and angioplasty restenosis.

14.   The system of claim 3, wherein the TLR-4 inhibitor is the nucleic acid encoding the soluble TLR-4 protein.

15.   The system of claim 14, wherein the soluble TLR-4 protein is unable to participate in normal TLR-4 signal transduction.

16.   The system of claim 14, wherein the soluble TLR-4 protein lacks a substantial portion of the normal TLR-4 signal transduction domain.

17.   The system of claim 14, wherein the soluble TLR-4 protein competes for a non-bound TLR-4 ligand.

18.   The system of claim 17, wherein the non-bound TLR-4 ligand is a chlamydial heat shock protein-60 (cHSP60) or a lipopolysaccharide (LPS).

19.   The system of claim 3, wherein the TLR-4 inhibitor is the nucleic acid expressing the dsRNA, and the dsRNA further includes:

        a sense strand further including approximately 21 nucleotides; and

        an antisense strand further including approximately 21 nucleotides.

20.   The system of claim 19, wherein the sense strand and the antisense strand are paired such that they possess a duplex region of approximately 19 nucleotides.

21.   The system of claim 19, wherein the sense strand and the antisense strand each further include an overhang at a 3'-terminus of approximately 2 nucleotides.

22.   The system of claim 21, wherein the sense overhang and the antisense overhang are symmetrical.

23.   The system of claim 21, wherein the antisense overhang comprises a UU 3'-overhang or a dTdT 3'-overhang.

24.   The system of claim 23, wherein the UU 3'-overhang or the dTdT 3'-overhang is complementary to the mRNA.

5   25.   The system of claim 21, wherein at least one of the sense overhang and the antisense overhang further includes a deoxythymidine.

26.   The system of claim 3, wherein the TLR-4 inhibitor is the protein sequence that corresponds to at least the portion of the receptor that binds to the TLR-4 ligand during the TLR-4 signal transduction event.

10   27.   The system of claim 26, wherein the receptor is a TLR-4 receptor or an MD2 receptor.

28.   The system of claim 26, wherein the protein sequence comprises from about 10 to about 20 amino acids.

29.   A method of treating a vascular disease, the method comprising the steps of:

15   providing a TLR-4 inhibitor; and

                        administering the TLR-4 inhibitor to a mammal in an amount effective to at least partially inhibit the biological activity of TLR-4.

30.   The method of claim 29, wherein the vascular disease is selected from the group consisting of atherosclerosis, transplant atherosclerosis, vein-graft atherosclerosis,

20   thrombosis, restenosis, stent restenosis, and angioplasty restenosis.

31.   The method of claim 29, wherein the step of administering the TLR-4 inhibitor further comprises administering the TLR-4 inhibitor in an amount effective to inhibit the vascular disease.

32.   The method of claim 29, wherein the step of administering the TLR-4 inhibitor further comprises administering the TLR-4 inhibitor intraveneously.

33.   The method of claim 29, wherein the step of administering the TLR-4 inhibitor further comprises administering the TLR-4 inhibitor intramuscularly.

34.   The method of claim 29, wherein the step of administering the TLR-4 inhibitor further comprises delivering the TLR-4 inhibitor with an intravascular device.

35.   The method of claim 34, wherein the intravascular device is a catheter or a stent.

36.   The method of claim 34, wherein the intravascular device is coated with the TLR-4 inhibitor.

37.   The method of claim 29, wherein the TLR-4 inhibitor is selected from the group consisting of a nucleic acid expressing antisense TLR-4 RNA, a nucleic acid encoding a soluble TLR-4 protein, a nucleic acid encoding a hammerhead ribozyme that cleaves TLR-4 mRNA, an antisense TLR-4 oligodeoxinucleotide (ODN), a nucleic acid expressing a double stranded RNA (dsRNA) that is sufficiently homologous to a portion of a TLR-4 gene product such that the dsRNA is capable of inhibiting the encoding function of mRNA that would otherwise cause the production of TLR-4, a protein sequence that corresponds to at least a portion of a receptor that binds to a TLR-4 ligand during a TLR-4 signal transduction event, and an anti-TLR-4 antibody.

38.   The method of claim 37, wherein the TLR-4 inhibitor is the nucleic acid expressing antisense TLR-4 RNA.

39.   The method of claim 37, wherein the TLR-4 inhibitor is the nucleic acid encoding the hammerhead ribozyme that cleaves TLR-4 mRNA.

40.   The method of claim 37, wherein the TLR-4 inhibitor is the antisense TLR-4 oligodeoxinucleotide (ODN).

41.   The method of claim 37, wherein the TLR-4 inhibitor is the anti-TLR-4 antibody.

42.   The method of claim 37, wherein the TLR-4 inhibitor is included within a vector.

43.   The method of claim 42, wherein the vector is selected from the group consisting of adenoviruses, adeno-associated viruses, retroviruses, lentiviruses, viral vectors, and non-viral vectors.

44.   The method of claim 42, wherein the vector is an adenovirus serotype 5-based vector.

45.   The method of claim 42, wherein the TLR-4 inhibitor is selected from the group consisting of a nucleic acid expressing antisense TLR-4 RNA, a nucleic acid encoding soluble TLR-4 protein, a nucleic acid encoding a hammerhead ribozyme that cleaves TLR-4 mRNA, and a nucleic acid expressing a double stranded RNA (dsRNA) that is sufficiently homologous to a portion of a TLR-4 gene product such that the dsRNA is capable of inhibiting the encoding function of mRNA that would otherwise cause the production of TLR-4.

46.   The method of claim 37, wherein the TLR-4 inhibitor is the nucleic acid encoding the soluble TLR-4 protein.

47.   The method of claim 46, wherein the soluble TLR-4 protein is unable to participate in normal TLR-4 signal transduction.

48.   The method of claim 46, wherein the soluble TLR-4 protein lacks a substantial portion of the normal TLR-4 signal transduction domain.

49.   The method of claim 46, wherein the soluble TLR-4 protein competes for a non-bound TLR-4 ligand.

50.   The method of claim 49, wherein the non-bound TLR-4 ligand is a chlamydial heat shock protein-60 (cHSP60) or a lipopolysaccharide (LPS).

51.   The method of claim 37, wherein the TLR-4 inhibitor is the nucleic acid expressing the dsRNA, and the dsRNA further includes:

        a sense strand further including approximately 21 nucleotides; and

        an antisense strand further including approximately 21 nucleotides.

52.   The method of claim 51, wherein the sense strand and the antisense strand are paired such that they possess a duplex region of approximately 19 nucleotides.

53.   The method of claim 52, wherein the sense strand and the antisense strand each further include an overhang at a 3'-terminus of approximately 2 nucleotides.

54.   The method of claim 53, wherein the sense overhang and the antisense overhang are symmetrical.

55.   The method of claim 53, wherein the antisense overhang comprises a UU 3'-overhang or a dTdT 3'-overhang.

56.   The method of claim 55, wherein the UU 3'-overhang or the dTdT 3'-overhang is complementary to the mRNA.

5   57.   The method of claim 53, wherein at least one of the sense overhang and the antisense overhang further includes a deoxythymidine.

58.   The method of claim 37, wherein the TLR-4 inhibitor is the protein sequence that corresponds to at least the portion of the receptor that binds to the TLR-4 ligand during the TLR-4 signal transduction event.

10   59.   The method of claim 58, wherein the receptor is a TLR-4 receptor or an MD2 receptor.

60.   The method of claim 58, wherein the protein sequence comprises from about 10 to about 20 amino acids.
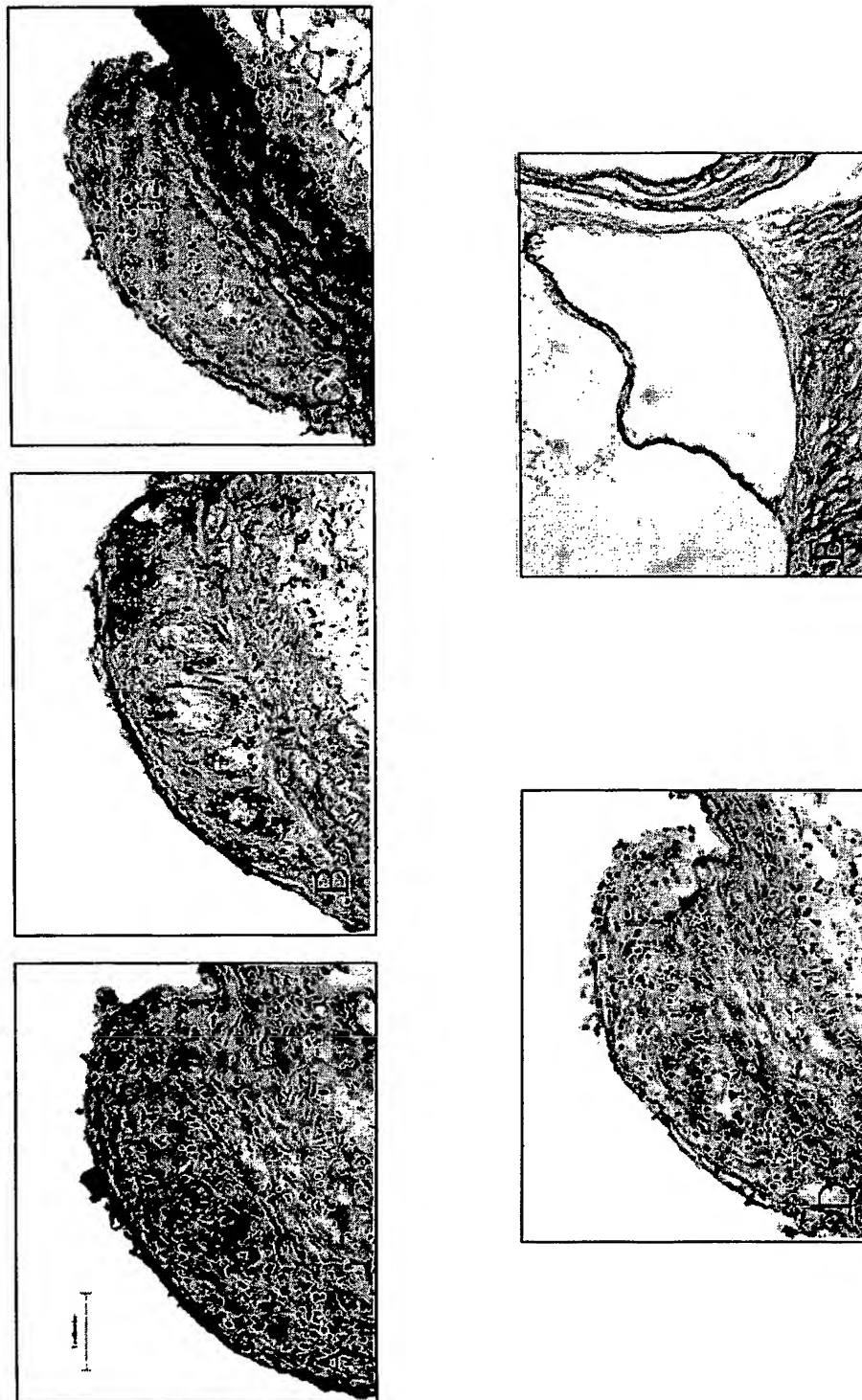
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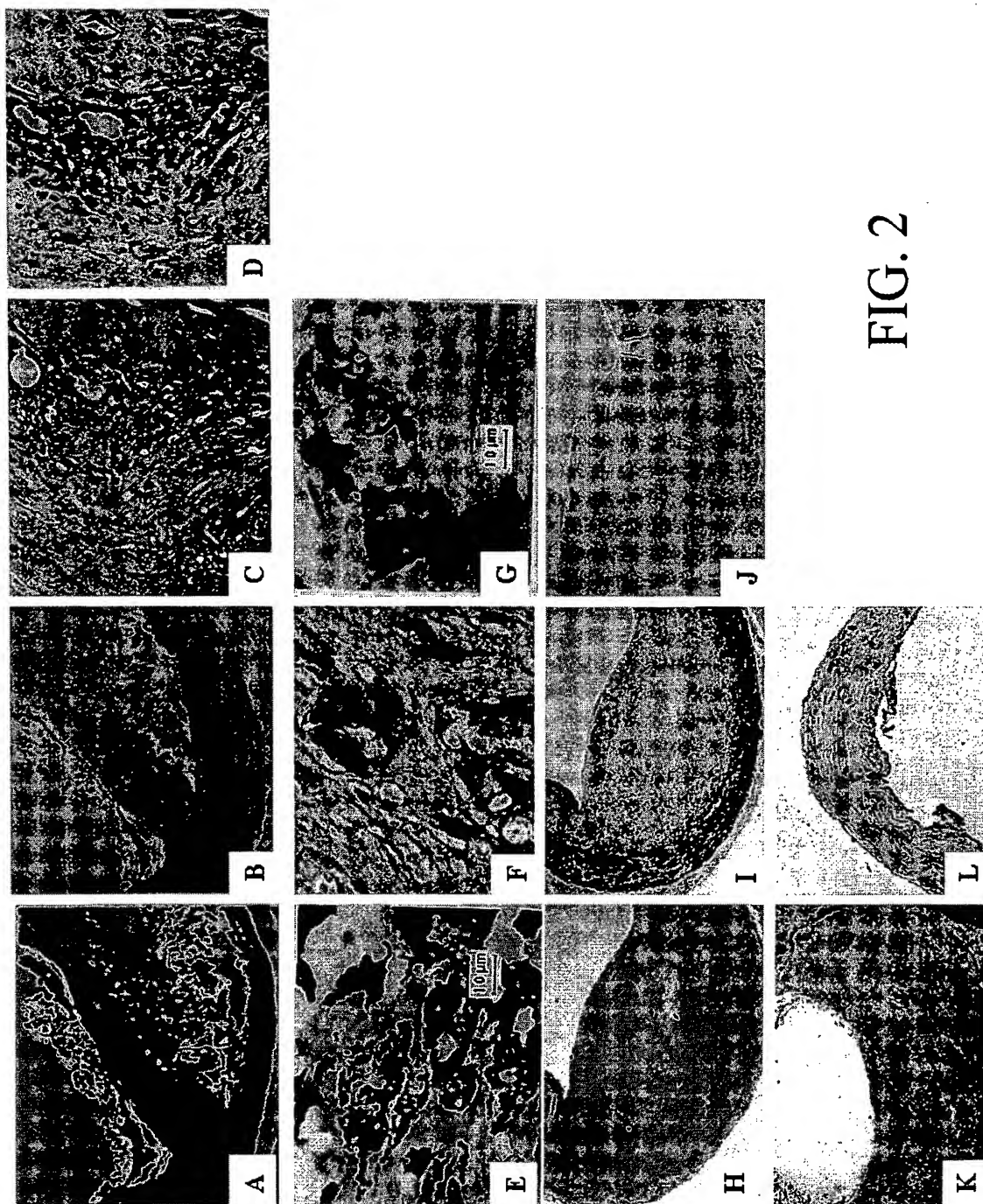
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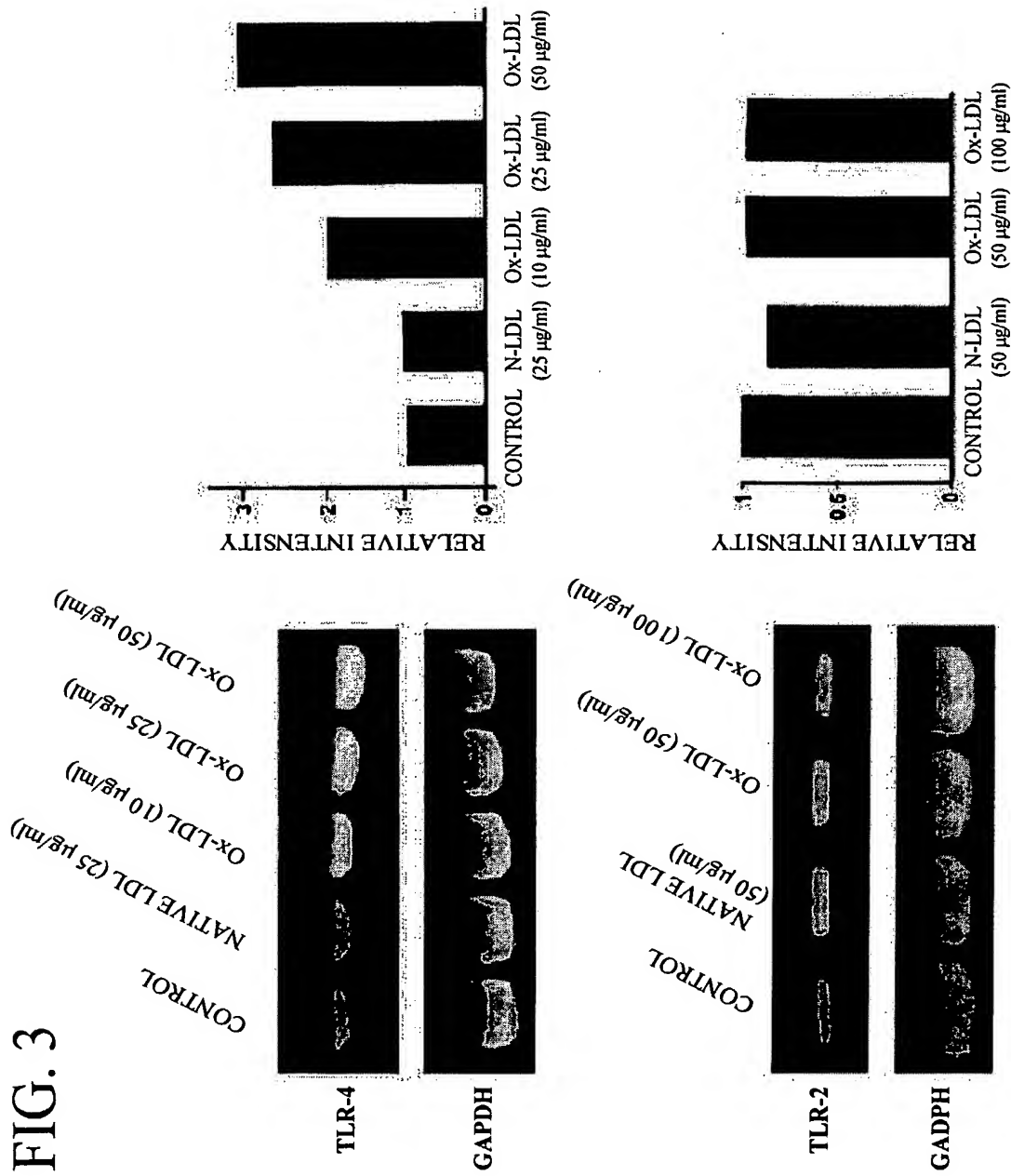
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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7   A61K45/00   A61L31/16   A61P9/10   A61K31/7105   A61K38/17
          A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7   A61K   A61L   A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

WPI Data, EPO-Internal, PAJ, MEDLINE, BIOSIS, CHEM ABS Data, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00 72023 A (SINGH MAHAVIR ;FUST GYORGY (HU); ROMICS LASZLO (HU); PROHASZKA ZOL) 30 November 2000 (2000-11-30)	29-60
Y	page 8, line 14 - line 24	1-28

X	WO 01 43691 A (KOLB HUBERT ;ELIAS DANA (IL); PEPTOR LTD (IL)) 21 June 2001 (2001-06-21)	29-60
Y	page 6, line 13 - line 16 page 12, line 9 - line 15; claims 19,20	1-28

X	WO 00 20019 A (HARATS DROR ;GEORGE JACOB (IL); SHOENFELD YEHUDA (IL)) 13 April 2000 (2000-04-13)	29-60
Y	page 10, line 20 - line 26	1-28

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[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

° Special categories of cited documents :

'A' document defining the general state of the art which is not considered to be of particular relevance

'E' earlier document but published on or after the International filing date

'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

'O' document referring to an oral disclosure, use, exhibition or other means

'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 February 2003	28/02/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Pilling, S

5

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 733 327 A (IGAKI KEIJI ET AL) 31 March 1998 (1998-03-31) column 2, line 54 -column 3, line 18; examples 1-7 ---	1-28
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